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THE ROLES OF LYMPHOTOXIN AND TUMOR NECROSIS
FACTOR-ALPHA IN THE PATHOGENESIS OF
EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

Sonia Friedman

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A Thesis Submitted to Yale University
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Abstract

THE ROLES OF LYMPHOTOXIN AND TUMOR NECROSIS FACTOR-ALPHA IN THE PATHOGENESIS OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS. Sonia

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Multiple sclerosis (MS) is a progressively debilitating inflammatory disease of the central nervous system (CNS). Although the etiology of MS is unclear, the cytokines tumor necrosis factor-alpha (TNF- α) and lymphotoxin (LT) have recently been implicated in its pathogenesis. To examine the roles of LT and TNF- α , we worked with experimental allergic encephalomyelitis (EAE), a murine model of MS in which the host animal is induced to mount an immune attack to myelin basic protein (MBP). EAE is characterized by invasion of the CNS with lymphocytes leading to demyelination histologically and paralysis clinically. We induced EAE in SJL/J mice by injecting LNC-8 or V β 17⁻ cells, MBP-specific CD4⁺ T cell lines known to secrete LT and TNF- α and derived from the popliteal lymph



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nodes of an SJL/J mouse immunized with porcine MBP. We used Di-I-C, a new and highly effective specific cell label, to explore the timing of T cell entry into the CNS and T cell migration and distribution within the CNS in EAE. We then used TN3.19.12, a newly developed hamster monoclonal anti-LT/TNF- α antibody, to block the clinical induction of EAE and to examine the histological effects of EAE alleviation. Our experiments showed that Di-I-C did not interfere with transfer of EAE; the average day of onset and average clinical disease score of mice injected with dyed cells (7.2 and 2.5) were comparable to those of mice injected with undyed cells (6.7 and 2.2). TN3.19.12, when injected 48 hours after dyed cells, alleviated severity of EAE (day of onset: 4.7, maximum score: 1.0). Histological analysis revealed Di-I-C labelled T cells throughout the brain and spinal cord 8 hours post injection (score = 2+). T infiltration peaked at 11 hours post injection (score= 4+), dwindled by 12 hours post injection (score = 1+) and disappeared by 18 hours post injection. Hematoxylin and eosin stained sections of brain and spinal cord from mice with advanced clinical disease showed marked perivascular infiltration of inflammatory cells. This infiltration was much reduced in antibody injected animals. These results suggest that clinical signs of EAE occur long after T cells

specific for MBP have entered and left the CNS. TN3.19.12 probably alleviates disease by blocking the actions of soluble LT and TNF- α and thus may be important in inhibiting inflammatory cell recruitment, and preventing alterations in blood brain barrier permeability.

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by primary destruction of myelin. It affects scattered areas of the CNS but most often plaques are seen in periventricular white matter, brainstem, spinal cord, and optic nerves. These plaques are characterized by destruction of myelin sheaths with preservation of axons and death of oligodendrocytes within the center of the lesion. During early plaque development, perivascular inflammatory cells such as lymphocytes, plasma cells, and macrophages pass through the blood brain barrier to invade the substance of the white matter and are thought to play a critical role in myelin destruction. In later plaque evolution, astrocytes produce extensive gliosis and oligodendrocytes undergo hyperplasia at the edges of the plaque (Raine et. al., 1981) and make ineffective attempts at remyelination (Rodriguez, 1989).

The pathogenesis of MS is unclear but the most widely considered hypothesis, the "autoimmune" theory, is that MS is the result of an immune reaction against self CNS antigens, particularly those present on oligodendrocytes, the cells in the CNS that make myelin. T cells are

thought to enter the CNS through endothelial cells of the blood brain barrier and to react with normal white matter. It is not known why in MS that previously harmless CD4⁺ T cells suddenly become activated against self. Since in MS lesions, endothelial cells and astrocytes are known to express class II MHC molecules, it is possible that they could present the myelin antigens to CD4⁺ T cells. These T cells would then be activated against self antigens and could recruit lymphokines and macrophages to help mediate myelin destruction (Rodriguez, 1989). It is not known why endothelial cells and astrocytes upregulate class II MHC molecules in MS and if they present self CNS antigens.

Another important hypothesis, called the "bystander" hypothesis is that myelin is an innocent bystander in MS and that it is destroyed as a consequence of an immune response occurring within the CNS. This hypothesis could explain why different viruses can induce demyelination in the nervous system of rodents (Rodriguez, 1989) and why etiologic agents such as measles virus (Haase et al., 1989) have reportedly been found in MS brains. Perhaps viruses or other infectious agents frequently invade the CNS and T lymphocytes and macrophages are recruited to

defend against infection. In the process of clearance of virus by T cells, myelin may be destroyed nonspecifically by lymphokines or neutral proteases released by activated macrophages (Rodriguez, 1989). In addition, there may be non-specific migration into the CNS by T cells activated against antigens not found in the CNS. Such T cells could cross react with CNS antigens. Also, the mode of egress of T cells from the CNS is ill-defined. If they cannot find their way readily out of the CNS parenchyma, T cells may continue to induce inflammation and myelin breakdown.

These theories about the pathogenesis of MS brush over two very complex issues. Since the CNS parenchyma of a healthy individual is an "immunologically priveleged" site with few lymphocytes (Traugott, 1985) and undetectable MHC molecules (Hickey, 1991), it is difficult to explain the detection of antigens and the initiation of inflammation within the CNS. First, it is unknown how and when lymphocytes cross the blood brain barrier and second it is unknown how lymphocytes and/or macrophages mediate inflammation and myelin destruction.

Recently, it has been suggested that the cytokines TNF- α and

lymphotoxin (LT; TNF- β) may play a role in the pathogenesis of several human neurologic diseases including MS. LT and TNF- α are closely related factors with extensive sequence homology and each has activities that could cause inflammation and demyelination. TNF- α is produced by macrophages after stimulation with lipopolysaccharide (LPS) and both LT and TNF- α are released by T cells activated by antigen or by infection with some viruses, including human T cell leukemia virus (HTLV-1) (Paul, 1988). Both LT and TNF- α have been associated with cachexia (Beutler, 1986) and TNF- α has been implicated in the pathogenesis of cerebral malaria. More importantly, TNF- α is cytotoxic for oligodendrocytes in culture and induces a delayed onset degradation of myelin in organotypic culture of nervous tissue (Selmaj et al., 1988). Oligodendrocytes in culture are even more sensitive to LT (Selmaj et al., 1991).

LT and TNF- α have also been detected in the brains of MS patients.

Anti-TNF- α mouse monoclonal and rabbit polyclonal antibodies label macrophages and astrocytes at MS lesion sites (Hofman et al., 1989). Selmaj et al. have shown for the first time evidence that LT, as well as

TNF- α , is present at MS lesions. In their most recent experiments, Selmaj et al. demonstrate that antibodies to LT react with CD3+ lymphocytes and microglial cells in active lesions whereas antibodies to TNF- α react with astrocytes and macrophages in active lesions and with occasional endothelial cells at the edges of acute lesions (Selmaj et al., 1991). Thus, many different cell types may secrete these cytokines and induce inflammation and demyelination at MS lesion sites.

Even more interesting is the fact that LT and TNF- α may initiate and even perpetuate the breakdown of the normally intact blood brain barrier. LT and TNF- α can interact with endothelial cells and activate the endothelium of postcapillary venules. If cerebral endothelial cells respond in this way, enhanced lymphocyte adhesion (Cavender et al., 1989), increased expression of adhesion molecules such as ICAM-1 (Pober et al., 1987) and increased permeability (Shijo et al., 1989) could occur at the blood brain barrier. LT and TNF- α can also activate monocytes and induce the synthesis of a variety of other cytokines such as granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor and in this way perpetuate the

immunopathological process (Selmaj et al., 1991).

Perhaps one of the most striking examples of the potential importance of LT and TNF- α in the pathogenesis of MS is the work of Ruddle et al. on experimental allergic encephalomyelitis (EAE). A murine model of MS, EAE is an autoimmune condition in which the host animal is induced to mount an immune attack to myelin basic protein (MBP), a major protein component of CNS myelin. It is characterized by invasion of the CNS with lymphocytes leading to demyelination histologically and paralysis clinically. The disease occurs after injection of the host animal with an emulsion of antigen (spinal cord components) or after passively transferring CD4⁺ T cells specific for MBP or proteolipid protein (Traugott et al., 1985). There is a limited heterogeneity of T cell receptors from CD4⁺ lymphocytes that mediate EAE with the majority of lymphocytes recognizing the N-terminal MBP-nonapeptide in association with I-A^U and using the VB8 gene element in PL/J mice. In SJL/J mice, most T lymphocytes recognize internal peptides and use VB4, VB17 and other gene elements. (Acha-Orbea et al., 1988).

In earlier *in vitro* experiments from the Ruddle lab that implicated a

role for LT and TNF- α in EAE, researchers used a series of MBP-reactive PL/J T cell clones that had identical antigen fine specificity and MHC restriction, used the same TCR V β gene and produced interleukin-2 (IL-2) in response to the encephalitogenic peptide (amino acids 1-11) of MBP presented in the context of H-2^U. Despite these similarities, the clones differed in their ability to transfer EAE. Clones that produced greater incidence of disease and greater severity of clinical signs secreted mRNA for LT and high levels of cytotoxic factors. Clones that did not transfer disease produced low or undetectable levels of cytotoxic activity and LT mRNA (Powell et al., 1990).

In subsequent *in vivo* experiments, Ruddle et al. demonstrated that inhibition of LT and TNF- α activity prevented clinical signs of paralytic disease (Ruddle et al., 1991). To block LT and TNF- α activity, they used TN3.19.12, a hamster monoclonal antibody that effectively neutralizes both exogenously produced LT and TNF- α in activated T cell culture supernatants and endogenously produced TNF *in vivo* (Sheehan, 1989). Ruddle et al. induced EAE in mice with LNC-8, an MBP-specific T cell line derived from the popliteal lymph nodes of an SJL/J mouse immunized

with porcine MBP. It was known that LNC-8 secreted both LT and TNF- α because culture supernatants from LNC-8 cells activated by ConA, a nonspecific mitogen, YCD3-1, an anti-CD3 antibody, and by MBP presented by irradiated syngeneic spleen cells were all cytotoxic to WEHI 164 cells, a target for both LT and TNF- α . The amount of cytotoxic activity in the culture supernatants increased over time of stimulation (measured at 8, 24, and 48 hours), especially when LNC-8 cells were first activated with MBP and irradiated spleen cells. LT and TNF- α mRNA was isolated from LNC-8 cells activated by all three methods. TN3.19.12 anti-LT/TNF- α antibody completely neutralized WEHI 164 cytotoxic activity of supernatants obtained after all methods of activation of LNC-8 cells including MBP + spleen cells, Con A, and anti-CD3+ antibody (Ruddle et al., 1991).

Next, and even more importantly, Ruddle et al. inhibited LNC-8 transfer of EAE by treatment of recipient mice with anti-LT/TNF- α antibody. In five different experiments, groups of 4 or 5 mice were injected intraperitoneally (IP) with $7.5-9 \times 10^6$ LNC-8 cells and then treated 48 hours later with either a single 300 μ g IP injection of 1)

TN3.19.12, 2) L2D39, a control hamster monoclonal antibody, or 3) PBS.

Mice were observed daily for three weeks to document onset of clinical signs of EAE. The clinical scale used to grade the severity of EAE was as follows: 0 = normal, 1 = tail limpness, 2 = paraparesis with a clumsy gait, 3 = hind limb paralysis, 4 = hind and fore limb paralysis, 5 = death. In all groups of mice, TN3.19.12 greatly reduced severity of disease and delayed disease onset compared to control antibodies. Whereas average disease scores for PBS and L2D39 injected animals were 2.6 and 2.2 respectively and average disease onsets were 6.2 and 7.8 days respectively, the average disease score for TN3.19.12 protected mice was 0.9 and the average disease onset was 8.6 days (Ruddle et al., 1991).

In their next experiment, Ruddle et al. produced even more clinically apparent disease. They injected 12×10^6 LNC-8 cells intravenously into groups of nine to ten mice and tried to block transfer at 48 hours post injection with 1 mg of 1) TN3.19.12, 2) anti-IL4 antibody, and 3) PBS. Average disease severity in the PBS and anti-IL4 treated animals was 3.9 and 3.3 respectively while average disease severity in the TN3.19.12 treated group was 0.9. The incidence of disease was also reduced from

89% in the PBS treated group to 20% in the anti-LT/TNF- α treated group.

Several groups of mice were observed for two to three months after TN3.19.12 injection and the inhibition of EAE transfer proved to be long-lived. No mice developed disease if it had not occurred by day 12 (Ruddle et al., 1991).

Histopathology correlated well with degree of clinical disease. On limited histological examination, there was little or no meningeal infiltration or inflammatory cells surrounding cerebral venules in animals where LT and TNF- α activity was blocked. All mice from the groups that received LNC-8 cells and control antibodies or PBS that were examined had histologic signs of perivascular cuffing and infiltration consistent with their clinical signs (Ruddle et al., 1991). Thus anti-LT/TNF- α antibody prevents both the clinical and histologic manifestations of EAE.

While other antibodies have been shown to block EAE, anti-LT/TNF- α antibodies like TN3.19.12 may play the greatest role in treating MS itself because it binds T cell mediators rather than T cells themselves. Sriram et al. suppressed passively transferred EAE with injections of anti-IA

antibodies (Sriram et al., 1987), Urban et al. blocked MBP peptide-induced EAE with an antibody to the V β 8 gene element of the CD4⁺ T cell receptor (Urban et al., 1988) and Rose et al. blocked passive transfer of EAE with IL2-PE40, a chimeric protein composed of human IL2 genetically fused to a modified form of *Pseudomonas* exotoxin lacking the cell recognition domain (Rose et al., 1991). In contrast to these previous experiments, TN3.19.12 does not inactivate all CD4⁺ T cells or even a major component of the T cell repertoire. Since LNC-8 is a cell line that contains at least two T cell populations that use different TCR β genes (Ruddle et al., 1991), it can also be seen that TN3.19.12 activity is not limited to a single T cell clone. The non-specificity is important because in MS, although the number of TCR β genes used may be limited, it is certainly greater than one (Ruddle et al., 1991). Thus, rather than binding to specific T cell lines, TN3.19.12 probably inhibits the activity of cytokine mediators that are produced *in vivo* by activation of these antigen-specific T cells.

However, the mechanisms by which this potentially very beneficial antibody inhibits the transfer of EAE remains unclear. It is possible that

TN3.19.12 could inhibit the disease in part by binding to and eliminating LT and TNF- α bearing cells but this does not seem very likely since TN3.19.12 does not react with the membrane bound form of TNF- α on macrophages. TN3.19.12 could also inhibit transfer of disease by preventing the activities of soluble LT and TNF- α at various stages. LT and TNF- α could directly affect the activities of transferred and activated T cells, macrophages, and additional cells within the CNS such as astrocytes, microglia, and oligodendrocytes. Alternatively, the interactions of transferred T cells, recruited T cells, and cells indigenous to the CNS may be altered. TN3.19.12 may also prevent LT and TNF- α induction of an increase in MHC determinants on endothelial cells (Poher et al., 1987), or alteration of CNS permeability and of influx of inflammatory cells through an increase in cell adhesion molecules on the endothelium (Poher et al., 1987). In addition, TN3.19.12 may neutralize cytokines secreted by inflammatory cells which cross the blood brain barrier and which directly affect the myelin sheath and influence viability of oligodendrocytes.

To understand exactly how anti-LT/TNF- α antibody blocks the

induction of EAE, it is first necessary to study the time course, histological pattern, and specificity of T cell migration into the CNS during EAE induction. Since the CNS of a healthy individual is normally "immunologically privileged" and contains very few native lymphocytes or expressed MHC molecules (Hickey, 1991), it is unclear how, when, and why T cells gain entry into the CNS and how they induce disease. In an elegant series of experiments, Hickey showed that regardless of their antigen specificity, their MHC restriction element, their MHC compatibility, or their phenotype, T cells can enter the CNS as long as they are activated. He injected 1) activated CD4⁺ T cells specific for MBP, 2) activated T cells with the same phenotype and using the same restriction element but specific for calf thymus histone, 3) activated pooled lymph node and splenic T cells of both CD4⁺ and CD8⁺ phenotypes and 4) resting pooled lymph node and splenic T cells into syngeneic rats and looked at infiltration into the CNS. The injected cells, whether specific for a CNS antigen or not, entered the CNS with the same kinetics and reached peak concentrations between 9 and 12 hours following injection. Resting lymphocytes never crossed the blood brain barrier. After twelve hours

post injection, the concentration of cells found in the CNS decreased, however, and only cells capable of recognizing their antigen among the proteins of the CNS, such as MBP-specific T cells, remained (Hickey, 1991).

One problem with this experiment, however, is that Hickey used a nonspecific T cell antibody to detect the injected T cells within the CNS. Since the rat normally contains very low but detectable levels of T cells in its CNS even without the injection of additional cells into the circulation, it is impossible to be certain that the cells injected into the circulation were the ones detected in the CNS. In fact, Hickey looked at spinal cords up to 72 hours post injection and found levels of presumed MBP-specific T cells to be only slightly greater than those of endogenous T lymphocytes (Hickey, 1991).

In an attempt to label only injected T cells, Hickey injected the same groups of Lewis cells into a DA rat and used a monoclonal antibody that detected only the Lewis class I (RT-IA) molecule. He thus followed the kinetics of Lewis cells in DA CNS and found that activated T cells will enter the CNS parenchyma rapidly with peak concentration between 9 and

12 hours and depart rapidly if they do not recognize their antigen. In this experiment, however, he could not determine whether MBP-specific T cells remained in the CNS after recognizing their antigen since the MHC of the DA rat is incapable of presenting any CNS antigen to Lewis lymphocytes in the correct MHC context. Hickey also did not look for lymphocytes in the brains of either Lewis or DA rats; he looked only at their spinal cords (Hickey, 1991).

Cross et al. used a different approach to examine the problem of T cell infiltration into the CNS. They used [^{14}C]thymidine to label MBP-sensitized lymphocytes from MBP-immunized mice before transferring them to naive syngeneic recipients. They looked at sections of brain and spinal cord 24 hours prior to and during initial clinical disease and their results confirmed what Hickey's suggested; they found a small number of labelled T lymphocytes around venules of the blood brain barrier up to two weeks after disease onset. However, at no time point did [^{14}C]thymidine labelled cells represent a sizable portion of the perivascular inflammatory cells commonly associated with clinical disease. Even in areas of extensive myelin pathology, radiolabelled cells

within the CNS were very rare. While there were many monocytes, macrophages, and PMNs surrounding venules, the mean percentage of labelled cells was 2.3% in the lumbar cord, 1.5% in the brainstem, and 0.5% in the brain parenchyma. When encountered, labelled cells were usually in close proximity to capillaries and small venules (Cross et al., 1990).

For controls, Cross et al., labelled cells from immunized mice with [^{14}C]thymidine following a three day stimulation period with purified protein derivative (PPD) of tuberculin prior to transfer. Mice did not become ill and no labelled cells were found in the CNS from days 5-27 post transfer. Since Hickey already suggested that activated T cells not specific for a CNS antigen may enter and leave the CNS within 48 hours, it is no surprise that Cross et al. did not find labelled PPD sensitive cells in the CNS after the fifth day post transfer (Cross et al., 1990). Thus, Cross et al. left out an essential part of this experiment- they did not look at tissue in the immediate period after T cell transfer.

Another problem with this experiment is the reliability of the label. Although Cross et al. suggest that MBP-specific cells act exclusively

from a perivascular location to orchestrate the influx of inflammatory cells (Cross et al., 1990), it is possible that the cells divide rapidly immediately upon entering the CNS parenchyma and dilute out the label. For how can T cells find their antigen if they only remain near the blood vessels? EAE pathology is not solely confined to perivenules; it is found deep in the brain and spinal cord parenchyma (Raine et al., 1980).

In our series of experiments, we attempt to address the technical problems of these previous experiments and study by histological analysis the induction of EAE and its alleviation by anti-LT/TNF- α antibody. We use a new and highly effective and specific cell label, Di-I-C to explore not only the timing of T cell entry into the CNS in EAE but also T cell migration and distribution within the CNS. This lipid soluble dye has never been used before in T cell homing experiments and it is extremely important since it partitions into the phospholipid bilayer and allows visualization of the entire cell. Since it labels the T cell for prolonged periods of time, it permits tracking of T cells within the CNS. This dye also permits, for the first time, studies of the mechanism of TN3.19.12 anti-LT/TNF- α antibody inhibition of EAE. In these

experiments, we demonstrate the effects of TN3.19.12 on T cell entry into the CNS and define a role for LT and TNF- α in EAE and possibly in MS itself.

Materials and Methods

Di-I-C Labelling

Di-I-C(18)3 or 1' dioctadecyl 3-3-3'-3'tetramethylenolcarbocyanine perchlorate was obtained from Molecular Probes Inc., P.O. Box 22010, 4849 Pitchford Avenue, Eugene, OR, 97402 (503-344-3007) cat# 282. 100µg units cost \$75.00. It was stored at -20°C in powdered form and wrapped tightly in aluminum foil to keep out light. It is a cationic membrane probe that partitions into the phospholipid bilayer and is non-invasive and non-toxic. It divides with the dividing cell and has stained MBP-specific F128 T cells in culture for up to two months with progressively diminishing signal (Ruddle et al., unpublished results). 800µg-1mg was dissolved per 1 ml of ethanol. The solution was agitated and heated in a 37°C water bath until the dye went into solution. 15µl of this solution was added to each ml of 5×10^6 cells + 10% fetal calf serum (FCS) + RPMI 1640 media. Cells fluoresced distinctly under UV with filters that excited rhodamine or fluorescein compounds.

LNC-8 and VB17- T Cell Proliferation Assay

This assay was performed to confirm that both LNC-8 and VB17⁻ T

cells could be stimulated with porcine MBP, irradiated syngeneic spleen cells, and recombinant IL-2. V β 17⁺ is a cell line derived from the LNC-8 cell line that secretes both LT and TNF- α and induces EAE just as effectively as LNC-8 cells (Clark et al., unpublished observation). Both types of T cells were plated at 2×10^6 per well with 10% FCS in 96 well plates and stimulated for 48 hours with either 10 μ g/ml porcine MBP + irradiated syngeneic spleen cells, 1 μ g/ml recombinant IL-2 + 10 μ g/ml porcine MBP + irradiated syngeneic spleen cells, or 5 μ g/ml Con A. Cells were then counted with a Coulter cell counter. LNC 8 and V β 17⁺ cells that were not stimulated with any of the above agents were grown for 48 hours in 10% FCS and used as controls.

T Cell Culture and Activation

LNC-8 and V β 17⁺ T cell lines reactive to MBP were obtained from Robert Clark (University of Connecticut) and activated every other week prior to injection. Cells were fed RPMI 1640 media + 10% FCS twice a week on Monday and Friday, recombinant IL-2 (1 μ g/ml; Amgen) every other Monday, and recombinant IL-2, porcine MBP (10 μ g/ml; Calbiochem) and irradiated syngeneic spleen cells every other Friday. Cells were

injected at intracardiac (IC) and intraperitoneal (IP) sites of SJL mice

Tuesday morning following Friday activation with MBP, spleen cells, and IL-2 and refeeding on Monday with IL-2.

T Cell Labelling and Injection

On Tuesday morning, cells were harvested from plates and flasks, spun down, resuspended in RPMI and 10% FCS, and counted. Cells were dyed in RPMI 1640 and 10% FCS at a volume of 5×10^6 /ml for one hour in a 37°C water bath. They were agitated once every ten minutes to keep the dye evenly distributed. Three washes in 1% PBS were performed. Cells were then spun down and concentrated to a volume of 50×10^6 /ml in 1% PBS for IC injection or 10×10^6 /ml in 1% PBS for IP injection. Depending on the experiment, mice received anywhere from 10 - 15×10^6 cells (1-1.5 ml IP or 0.2 ml IC) IC injection was done free hand. Undyed cells, injected in equal volume and numbers, were used as controls. To determine if T cells transferred dye to spleen cells in culture, dyed LNC-8 and VB17⁻ cells were cultured with undyed, irradiated spleen cells for 1 week. Cells from the cultures were then examined under a fluorescence microscope.

Transfer of EAE

Two separate experiments were performed to test the effectiveness of LNC-8 and VB17⁺ T cell induction of EAE with and without dye. 14 female, 3 month old, SJL/J mice were used in each experiment and all animals were evaluated for clinical signs of EAE daily for at least three weeks after injection of cells. The clinical scale was as follows: 0 = normal, 1 = limp tail, 2 = paraparesis with a clumsy gait, 3 = hind limb paralysis, 4 = fore and hind limb paralysis, 5 = death. Animals used for histology were taken from these two experimental groups and 4 other experimental groups of 2-4 animals each described below. Results were compared by two-tailed T tests.

TN3.19.12 Antibody Injection

3 mice from the two large experimental groups were injected IP with 1 mg of TN3.19.12 anti-LT/TNF- α antibody in 0.5 ml of 1% PBS 48 hours after T cell injection. TN3.19.12 was injected only into mice which had received dyed cells. The antibody is as described in (Sheehan et al., 1989). Briefly, monoclonal antibody to murine TNF was produced after immunization of Armenian hamsters with purified E.Coli-derived

rMuTNF- α . Antibody from clone TN3.19.12 was purified and was found to inhibit 100% of the lytic activity of either recombinant or natural MuTNF- α at an antibody input of 25 ng/U. TN3.19.12 also inhibited all the lytic activity in culture supernatants from a variety of T cell sources, including activated T cell clones and T cell hybridomas (all of which expressed high levels of TNF- α and LT mRNA). Western blot analysis was used to document the physical form(s) of MuTNF recognized by TN3.19.12. Recombinant and macrophage-derived TNF displayed identical patterns of a single band with M_r 17 kDa. In contrast, T cell culture supernatants exhibited patterns consisting of two bands with M_r 17 and 24.7 kDa, the latter consistent with that of LT. These data suggest that TN3.19.12 recognizes both MuTNF- α and LT (Sheehan et al., 1989)

Histology and Tracking of Dyed T Cells

In addition to the two large experimental groups, four other experimental groups of 2-4 SJL mice each were used for histology and tracking of dyed T cells. Mice were killed 8 hours (1 mouse), 9 1/2 hours (1), 11 hours(2), 12 hours (1), 13 hours (2), 14 hours (1), 18 hours (2), 2

days (2), 1 week (1), 10 days (2), or two weeks (2) after dyed LNC-8 or VB17⁻ T cell injection and/or TN3.19.12 antibody injection. All mice killed one week or later post injection had clinical disease scores of at least 2.0. Mice were anesthetized with metofane and perfused with 10% formalin or 4% paraformaldehyde in 1% PBS. Brain, spinal cord, lymph nodes, spleen, liver, kidney, and lungs were removed and kept in 10% formalin or 4% paraformaldehyde for 1 day at 4°C. Organs were then placed in a 20% sucrose solution in 1% PBS and kept at 4°C for half a day. Tissue was then frozen in Tissue Tek and 10-15µm sections were cut on a cryostat and examined under filters that excite rhodamine and/or fluorescein light for presence and distribution of T cells. Amount of T cells per high power field were qualitatively scored from 1+ to 4+. Some brain and spinal cord sections were stained with hematoxylin and eosin and examined for overall morphology and for the presence of cellular infiltrates.

Results

LNC-8 and VB17⁻ Cell Proliferation Assay

Although both LNC-8 and VB17⁻ cells have both been shown to proliferate in response to MBP + IL-2 + spleen cells (Ruddle et al., 1990, Clark et al., unpublished results), this assay was done to determine whether our culture and activation methods were adequate. First, proliferation of LNC-8 and VB17⁻ cells in response to various stimulating agents was measured against proliferation of unstimulated LNC-8 and VB17⁻ cells (Table 1). On the average, LNC-8 cells stimulated with either MBP + spleen cells or IL-2 + MBP + spleen cells proliferated 279x more than unstimulated LNC-8 cells. VB17⁻ cells stimulated with either MBP + spleen cells or IL-2 + MBP + spleen cells proliferated 188x more than unstimulated VB17⁻ cells. LNC-8 and VB17⁻ cells stimulated with Con A rather than MBP + spleen cells + IL-2 only proliferated 25x and 30x more than non-stimulated LNC-8 and VB17⁻ cells.

T Cell Dyeing

LNC-8 and VB17⁻ cells were successfully dyed with Di-I-C. As shown in Figure 1, Di-I-C covers the entire cell and is easily visualized

under a filter that excites fluorescein light. This cell is from a one week culture and has been transferred from the culture dish onto a slide. As noted in Materials and Methods, Di-I-C stains dividing cells in culture and continues to stain cells at least up to two months in culture. In addition, Di-I-C does not transfer its label to spleen cells in culture. Figure 2 shows LNC-8 cells which have been dyed and added to irradiated spleen cells in culture. This photomicrograph was taken after two weeks in culture. While dyed LNC-8 cells fluoresce very brightly under a rhodamine filter, spleen cells are much smaller than T cells and fluoresce minimally or not at all. Thus, if it were possible that spleen cells inadvertently accompanied T cells across the blood brain barrier, they would not be visualized with this dye.

Transfer of EAE

These experiments showed that Di-I-C did not interfere with transfer of EAE. The average day of onset and average maximum clinical score of mice injected with dyed LNC-8 or V β 17⁻ T cells were comparable to those of mice injected with undyed cells (Table 2). Of 25 animals injected with either LNC-8 or V β 17⁻ T cells and no TN3.19.12

antibody in two separate experiments, the average day onset of disease for dyed cells injected IC (8 animals) was 4.0, for dyed cells injected IP (8 animals) was 7.2 ($p < 0.010$), and for undyed cells injected IC and IP (9 animals) was 6.7. (There was no statistically significant difference between dyed cells injected IP and undyed cells injected IC & IP). The average maximum score for dyed cells injected IC was 5.0, for dyed cells injected IP was 2.5 ($P < 0.010$), and for undyed cells injected IC and IP was 2.2. (There was no statistically significant difference between dyed cells injected IP and undyed cells injected IC & IP). Thus Di-I-C did not interfere with onset or severity of disease. Mode of injection however, did influence the transfer of EAE. T cells injected IC rather than IP induced disease more quickly and with greater severity.

TN3.19.12 Antibody Injection

To make certain that TN3.19.12 anti-LT/TNF- α alleviates the severity of EAE induced by dyed T cells, 3 animals were injected with TN3.19.12 IP 48 hours after dyed T cell IC injection. Results were exactly as demonstrated by Ruddle et al. 1990 with undyed T cells; TN3.19.12 reduced the clinical grade of EAE and slightly delayed the day

of disease onset (Table 2). Average day of onset with TN3.19.12 antibody injection was 4.7 ($P < 0.050$ when compared with dyed cells injected IC without antibody) and average maximum disease score was 1.0 ($P < 0.010$ when compared with dyed cells injected IC without antibody).

Histology and Tracking of Dyed T Cells

The most important part of this experiment is the qualitative evaluation of T cell migration and distribution within the CNS. To study this, we examined the brain and spinal cords of mice at various intervals after dyed T cell injection IP and/or IC. Mice used for time points of 8 hours (1), 9 1/2 hours (1), 11 hours (2), 12 hours (1), 13 hours (2), 14 hours (1), 18 hours (2) and 2 days post injection (2) were taken from 4 experimental groups of 2 to 4 animals each and mice used for 1 week (1), 10 day (2), and two week (2) time points were taken from the two large experimental groups of 14 animals each.

At eight hours post injection, numerous brightly labelled T cells were seen throughout the brain and spinal cord (score = 2+). Figure 3 shows the spinal cord of an animal killed 8 hours post injection of dyed VB17⁻ T cells examined under a filter that excites rhodamine light. A

brightly dyed T cell is seen in the spinal cord parenchyma. More dyed T cells are seen throughout the brain and spinal cord at 9 1/2 hours post injection (score = 3+). Figure 4a shows dyed V β 17⁺ T cells throughout the brain parenchyma and Figure 4b shows a dyed V β 17⁺ T cell in the spinal cord parenchyma of mice killed 9 1/2 hours post injection. These cells are seen under a filter that excites rhodamine.

Upon qualitative analysis, T cell infiltration into the CNS seems to peak at 11 hours post injection (score = 4+). Figure 5a shows dyed LNC-8 cells in the brain of a mouse killed 11 hours post injection. Here, there are numerous dyed cells within the brain parenchyma. Di-I-C outlines their membranes so well that one can almost see them "walking" through the brain, in active pursuit of their antigen, myelin basic protein. Figure 5b shows an LNC-8 cell at high magnification most likely in this active process of tracking down its specific antigen.

The number of T cells in the brain and spinal cord gradually dwindle at 12, 13, and 14 hours post injection. Figure 6 shows a mouse brain at 12 hours post injection of dyed V β 17⁺ cells under a filter that excites rhodamine light. The number of cells is diminished at this time in

comparison with previous times (score = 1+). At 18 hours post injection, no dyed T cells can be seen in the brain and spinal cord parenchyma. Accordingly, no dyed T cells could be found within the brain or spinal cords of mice killed at 2 days, 1 week, 10 days or 2 weeks post injection. Dyed T cells could be found in other organs of mice killed up to 2 weeks post injection, however. Numerous dyed T cells were seen in the lymph nodes, lungs, liver, and spleen of mice killed anytime from 8 hours post injection to 2 weeks post injection. This result implies that Di-I-C did not fade or dilute its label with cell division but rather that most or all labelled MBP-antigen specific T cells were no longer present within the CNS just 6 hours after they arrived.

Another important point is that TN3.19.12 probably does not work by eliminating T cells bearing LT and TNF- α because the antibody was injected 48 hours after injection of T cells, long after they had infiltrated into and apparently left the CNS. Thus, TN3.19.12 most likely interacts with soluble forms of LT and TNF- α and prevents their activities at various stages of disease transfer.

To examine the histological effects of TN3.19.12, several sections of

brain and spinal cord from mice with advanced clinical disease and from mice who had received the TN3.19.12 antibody were stained with hematoxylin and eosin and examined under a light microscope at 1 week, 10 days, and 2 weeks post injection. While there was marked perivascular infiltration of inflammatory cells in the brains and spinal cords of mice that did not receive the antibody, this infiltration was much reduced in antibody-injected animals. Thus, we demonstrate that TN3.19.12 blocks recruitment of macrophages, neutrophils and other inflammatory cells that may play a large part in EAE induction. As reported earlier by Ruddle et al. in their EAE TN3.19.12 antibody study, (Ruddle et. al., 1991) the histological picture of EAE and its alleviation by TN3.19.12 anti-LT/TNF- α antibody correlates well with the clinical disease process.

Table 1: T Cell Proliferation Assay

<u>Cell Type</u>	<u>Stimulating Agent</u>	<u>Proliferation Ratios</u>
LNC-8	MBP+SC	251
LNC-8	MBP+SC+IL-2	307
LNC-8	Con A	25
VB17 ⁻	MBP+SC	174
VB17 ⁻	MBP+SC+IL-2	201
VB17 ⁻	Con A	30

This table demonstrates the response of LNC-8 and VB17⁻ cells to various stimulating agents. LNC-8 and VB17⁻ cells were incubated in 10% FCS and stimulated for 48 hours with either 10µg/ml porcine MBP + irradiated spleen cells, recombinant IL-2 + 10µg/ml porcine MBP + irradiated spleen cells, or 5µg/ml Con A. Non-stimulated LNC-8 and VB17⁻ cells were used to establish baseline incorporation for the proliferation ratios.

Table 2: Clinical Evaluation of EAE Transfer

<u>Cells Injected</u>	<u>Average Day Onset</u>	<u>Average Maximum Score</u>
Dyed cells IC (8 animals)	4.0	5.0
Dyed cells IP (8 animals)	7.2	2.5
Undyed cells IC&IP (9 animals)	6.7	2.2
Dyed cells IC with TN3.19.12 antibody (3 animals)	4.7	1.0

This table demonstrates the average day onset and average maximum score of dyed T cells injected IC, dyed cells injected IP, undyed cells injected IC and IP, and dyed cells injected IC with TN3.19.12 antibody. When average day onset and average maximum score of dyed cells injected IC and dyed cells injected IP were compared by two-tailed t tests, a p value of <.010 was obtained. $p < .010$ when dyed cells injected IC with TN3.19.12 antibody were compared to dyed cells injected IP, $p < 0.05$ when average day onset of dyed cells injected IC with and without TN3.19.12 were compared, and $p < .010$ when average maximum score of dyed cells IC with and without TN3.19.12 were compared. There was no statistically significant difference between dyed cells injected IP and undyed cells injected IC&IP.

Figures



Figure 1

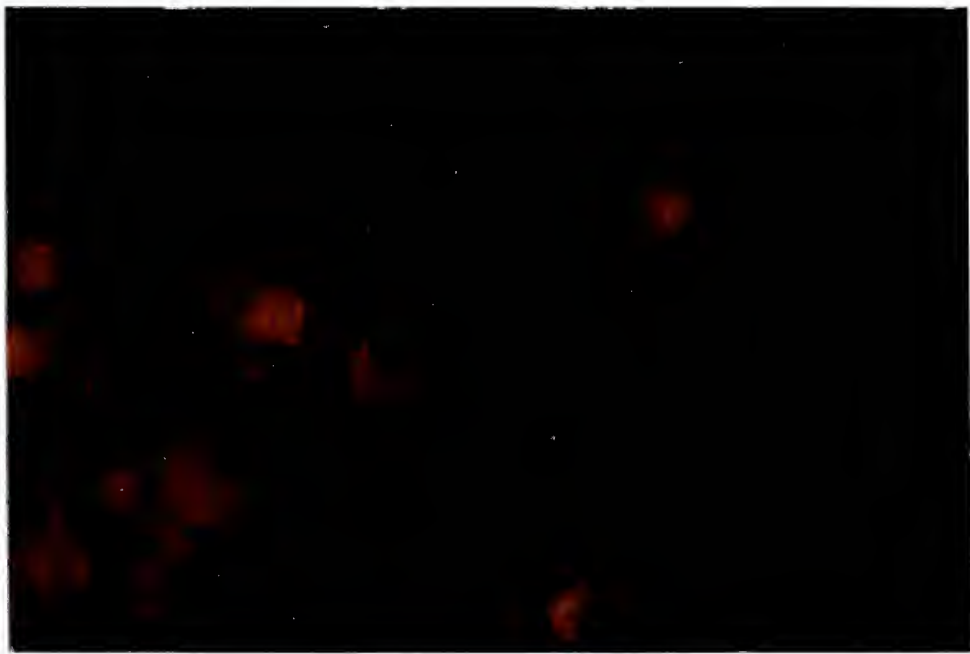


Figure 2



Figure 3



Figure 4a



Figure 4b

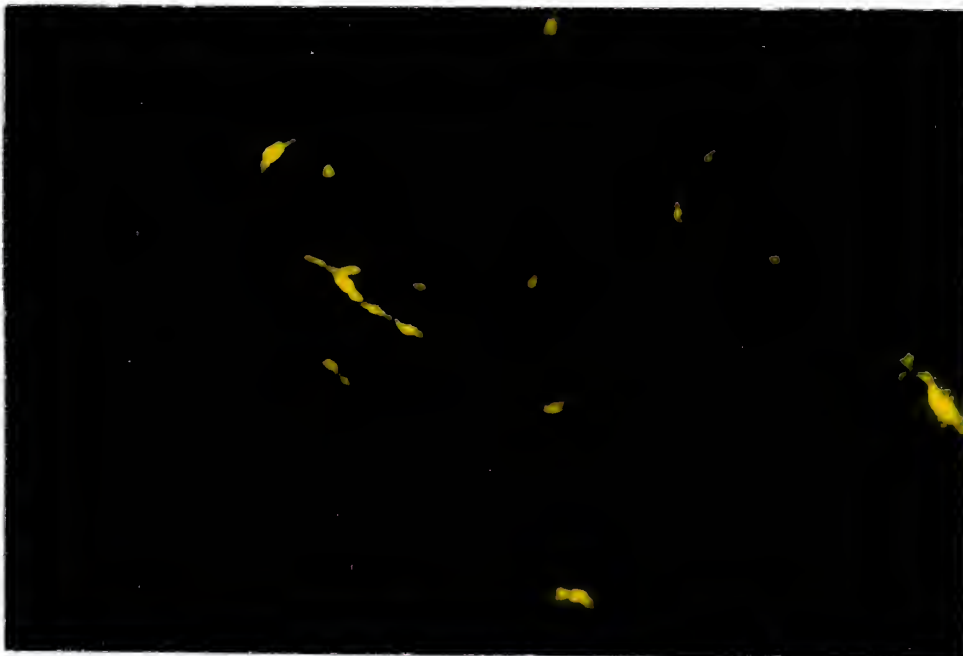


Figure 5a

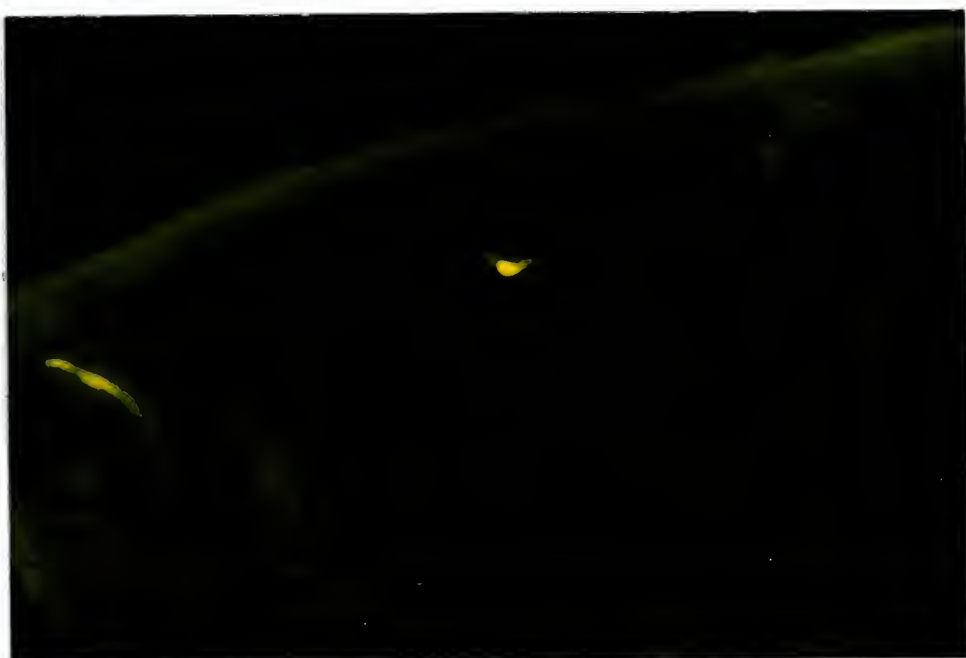


Figure 5b



Figure 6

Figure Legends

Figure 1: LNC-8 cell that has been dyed with Di-I-C and kept in culture for one week. Photographed under a filter that excites fluorescein. Magnification = 25X.

Figure 2: LNC-8 cells that have been dyed with Di-I-C, added to irradiated spleen cells in culture, and kept in culture for two weeks. Photographed under a filter that excites rhodamine. Magnification = 40X.

Figure 3: Spinal cord of an animal killed 8 hours post injection of VB17⁻ cells dyed with Di-I-C. Photographed under a filter that excites rhodamine. Magnification = 25X.

Figure 4a: Brain of a mouse killed 9 1/2 hours post injection of VB17⁻ cells dyed with Di-I-C. Photographed under a filter that excites rhodamine. Magnification = 10X.

Figure 4b: Spinal cord of a mouse killed 9 1/2 hours post injection of VB17⁻ cells dyed with Di-I-C. Photographed under a filter that excites rhodamine. Magnification = 25X.

Figure 5a: LNC-8 cells dyed with Di-I-C in the brain of a mouse killed 11 hours post injection. Photographed under a filter that excites fluorescein. Magnification = 25X.

Figure 5b: LNC-8 cells dyed with Di-I-C in the brain of a mouse killed 11 hours post injection. Photographed under a filter that excites fluorescein. Magnification = 25X.

Figure 6: VB17⁻ cell dyed with Di-I-C in the brain of a mouse killed 12 hours post injection. Photographed under a filter that excites rhodamine. Magnification = 10X.

Discussion

In these experiments, we demonstrate for the first time a highly effective way to visualize T cell entry into the CNS and to document histologically the events preceeding the induction of EAE. We use a T cell dye that does not interfere with transfer of EAE in any way and allows us to time T cell homing to the CNS and observe T cell migration within the CNS. We also show that most likely the anti-LT/TNF- α antibody, TN3.19.12 does not work by binding to antigens on the surface of specific T cells but rather alleviates clinical and histological signs of disease by binding to soluble forms of LT and TNF- α .

Di-I-C has never before been used in T cell homing experiments and is a much better cell marker than radionucleotide labels or nonspecific T cell antibody markers that label T cells only in histological sections of brain and spinal cord. It divides with the dividing cell and does not dilute out as radionucleotide labels often do (Wojcieszyn et al., 1983). After two weeks in vivo, it was visible in the lymph nodes, lungs, liver and spleen of mice and it was visible in vitro after two months in culture. It does not transfer to spleen cells in culture and outlines nicely the entire cell

membrane so one can follow T cell movement within the CNS. Moreover, it does not interfere with the induction of EAE or the alleviation of EAE by the TN3.19.12 antibody. It is also inexpensive, non-toxic and easy to use. Thus, it will be very helpful in future EAE and T cell homing experiments.

Due to the effectiveness of Di-I-C, we were able to define accurately the timing of T cell migration into the CNS. Unlike Cross et al. who failed to look at tissue in the immediate period after T cell transfer, we looked at CNS tissue from 8 hours post injection to two weeks post injection. Almost 5 days before their first time point, T cell infiltration into the CNS had already peaked and subsided. In addition, while Cross et al. found labelled cells only in close proximity to capillaries and small venules, we found them scattered evenly throughout the brain and spinal cord parenchyma. Perhaps if Cross et al. had looked earlier, they too would have found numerous scattered cells throughout the CNS. It is unclear why we did not find any residual cells in the CNS after 14 hours post injection. Although our cells could somehow have lost their label, this is not likely because dyed cells were seen in many other parts of the body including lymph nodes, lungs, liver, and spleen up to two weeks post injection.

Since Cross et al. found some T cells in the brain and spinal cord, albeit very few (Cross et al., 1991), it is surprising that our technique did not locate them. Likewise, Hickey et al. also found T cells in spinal cords up to 72 hours post injection. We have no way of telling in his case however, whether or not these T cells were host-derived since he did not label T cells before he injected them but rather used a non-specific T cell antibody on fixed spinal cord slices. Nonetheless, a larger and more detailed histological analysis of CNS tissue is needed with meticulous studies of brain and spinal cord for lingering MBP-specific T cells.

Di-I-C also enabled us to demonstrate T cell movement within the CNS- something neither Hickey nor Cross examined due to their cell label constraints. In Figures 3 through 6, one can see the MBP-specific T cells tracking through the brain and spinal cord, probably looking for their correct antigen. This picture correlates well with the idea that T cells having various specificities are able to cross the blood brain barrier. Yet only those that track down their specific antigens are able to remain within the CNS and to initiate and/or orchestrate the disease process. A combination of Di-I-C and a marker for blood vessels will facilitate

further studies of T cell movement within the CNS.

One helpful experiment in further studies would be to examine non-MBP-specific T cell migration into the CNS. It would be important to see whether non-MBP-specific T cells would home to the CNS and if they would leave soon after failing to locate their antigen. Hickey predicts this phenomenon in his review article; yet he cannot properly test it because he does not use a specific T cell label. Another new dye, Di-O, which has similar chemical properties to Di-I-C but is best visualized under a fluorescein filter, will enable us to label two separate T cell populations at the same time and to look at their differing kinetics. By labelling non-MBP-specific T cells with Di-O and LNC-8 or VB17- cells with Di-I-C, we could determine if T cells do stay in the CNS once they find their correct antigen or if a second wave of infiltration by specific T cells occurs.

Another issue that needs to be addressed in further studies is the nature and timing of the inflammatory cell recruitment. It is important to know when and how neutrophils and macrophages cross the blood brain barrier and what role they play in the disease process. This could be

accomplished by staining every other CNS section with hematoxylin and eosin and/or antibodies to inflammatory cells. We could then quantify the effects of TN3.19.12 on CNS inflammation. For example, TN3.19.12 may prevent alteration of CNS permeability by LT and TNF- α , it might prevent specific cell types from being recruited into the CNS or it might reduce LT and TNF- α -induced myelin breakdown.

TN3.19.12 might also prevent a LT and TNF- α -mediated increase of cell adhesion molecules on the endothelium of vessels of the blood brain barrier. To examine this possibility, we can study the regulation and expression of ICAM and other adhesion molecules in EAE. This would be fairly straightforward to do within our experimental design. We could simply block the expression of ICAM and other adhesion molecules with monoclonal antibodies and then monitor the inflammatory cell influx histologically. We could also look clinically for alleviation of disease. In addition, we could study how TN3.19.12 affects adhesion molecule expression.

We can add another piece to this puzzle by discovering when and how LT and TNF- α themselves are secreted in EAE. Specific T cells may

secrete LT and TNF- α in response to interacting with their proper CNS antigen. Macrophages and neutrophils may secrete TNF- α as part of their general inflammatory response. Other resident CNS cells may also secrete LT and TNF- α after activation. It is easiest to study the secretion of LT and TNF- α by examining the stages in which TN3.19.12 anti-LT/TNF- α antibody blocks EAE transfer.

In conclusion, these studies have brought us closer to understanding the process of EAE. We now understand when T cells specific for MBP cross the blood brain barrier and when they are present within the CNS in greatest numbers. We also now know that TN3.19.12 probably alleviates disease by blocking the actions of soluble LT and TNF- α . Since TN3.19.12 is so effective and is non-specific, an antibody like it generated against human tumor necrosis factors may be very helpful in the treatment of MS. Thus, we have not only begun to piece together the exact mechanism of EAE, but we can also use this knowledge to help treat human patients.

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